Presence of Epstein–Barr Virus in Langerhans Cells of CTCL Lesions

To the Editor:

The etiology of cutaneous T cell lymphomas (CTCL) remains unknown, so the involvement of viral agents and among them, Epstein–Barr virus (EBV), is suspected in the development of this pathology (Lee *et al*, 1990; Anagnostopoulos *et al*, 1996; Chang *et al*, 1998). Thus, it has been shown that keratinocytes of skin lesions of CTCL expressed EBV proteins in the skin of CTCL patients and that this detection was specific to this pathology (Dreno *et al*, 1994). Moreover, a significant increase of the titer of anti-EBV antibodies, particularly viral capsid antigen (VCA) antibodies, in CTCL patients compared with healthy controls, has been demonstrated (Jumbou *et al*, 1997).

Because of the observations mentioned above, we have focused on the nature of the EBV-expressing cells in these CTCL lesions.

Six CTCL patients were included in this study: 5 Sezary syndromes and 1 Mycosis fungoides stage IIb (Table I). The study was conducted according to Declaration of Helsinki Principles and the medical ethical committee of Nantes University Hospital approved all described studies. Patients gave written informed consent. The diagnosis of CTCL was confirmed by histology and immunochemistry. Moreover, a dominant Vβ T cell clone was identified both in the blood and in the skin of CTCL5 (Vβ13.6) and CTCL6 (Vβ2). All patients except one were serologically positive for EBV. Six normal skin samples, six atopic dermatitis inflammatory skin samples and six squamous cell carcinomas of immunosuppressed patients were used as controls. Cutaneous sections were obtained from paraffin-embedded biopsies from lesional skin of each patient and immunostained for EBNA2, late membrane protein (LMP), CD3 (all from DAKO, Trappes, France), and CD1a (Beckman Coulter, Marseille, France) using the indirect immunofluorescence technique as previously described. Moreover, a method combining *in situ* hybridization (ISH) and immunochemistry was performed on the same CTCL biopsies. The ISH was performed using FITC-conjugated EBER-1 or BHLF-1 (*Bam*HI fragment of lower stranded frame) PNA probes (PNA ISH detection kit, DAKO).

Concurrently, for the two patients with a dominant T cell clone, the cells were isolated with the antibody reactive with the TCR-Vβ-chain overexpressed (Beckman Coulter), and sorted according separation procedure from Miltenyi Biotec (Paris, France). Then total RNA was extracted from Vβ+ clones and non-sorted PBMC using the Trizol chloroform method, and cDNA was prepared by standard methods using reverse transcriptase and oligo-dT primer. The reaction cycle consisted of denaturation for 1 min at 94°C, annealing for 1 min at 60°C for EBV probes, at 55°C for β-actin and then extension for 1 min at 72°C, and was repeated 30 times. Positive control was the Akata cell-line, derived from a Burkitt's lymphoma patient.

By immunochemistry, EBNA2 protein was identified in epidermal cells of five of six skin lesions (Fig 1*A*), whereas no labelling was obtained in normal control skin, in atopic dermatitis skin, and in immunosuppressed patients' skin ($p = 0.02$, Fisher exact test, OR (odd ratio) infinite). Non-involved skin of CTCL patients have also been tested and we found an expression of EBNA2 in the epidermis as in lesional skin. No LMP expression was observed in the skin lesions of six CTCL patients. To identify the phenotype of EBV-expressing cells in epidermis, a double staining has been performed showing that only CD1a-positive cells were EBNA2 positive (Fig 1*B* and *C*). The mean percentage of infected cells (CD1a+ EBNA2+) in epidermis among three fields ($\times 40$) examined was $16\% \pm 3\%$. Moreover, the methodology combining ISH and immunochemistry showed that CD1a-positive cells were also EBER1 positive (Fig 1*D*), but not BHLF positive. There was no difference according the stage of the illness.

For the two patients with a dominant T cell clone, Vβ+ cells were sorted from blood and an EBV RT-PCR was performed on the extracted RNA. We found no expression of the EBV genome among neither Vβ+ cells, nor non-sorted PBMC (Fig 1*E*).

In summary, our study revealed that positive EBNA2 cells are present in the epidermis of five of six CTCL biopsies,

Table I. Correspondence between number of the patient, circulating Sezary cells, detection of a phenotypic Vβ dominant T cell clone and EBV serology

Patient	Circulating Sezary cells (%)	Vβ dominant[a]	Serology EBV (IgG, U per mL)	Stage of the disease
CTCL1	0	NF	1900	MF IIb
CTCL2	60	NF	Neg	SS
CTCL3	80	NF	130	SS
CTCL4	56	NF	82	SS
CTCL5	97	Vβ13.6	370	SS
CTCL6	75	Vβ2	1100	SS

[a]Determined by flow cytometry.
EBV, Epstein–Barr virus; CTCL, cutaneous T cell lymphoma; NF, not found with the panel of antibodies used; MF, mycosis fongoïde; SS, Sezary syndrome.

Abbreviations: CTCL, cutaneous T cell lymphoma; EBV, Epstein–Barr virus; LC, Langerhans cell

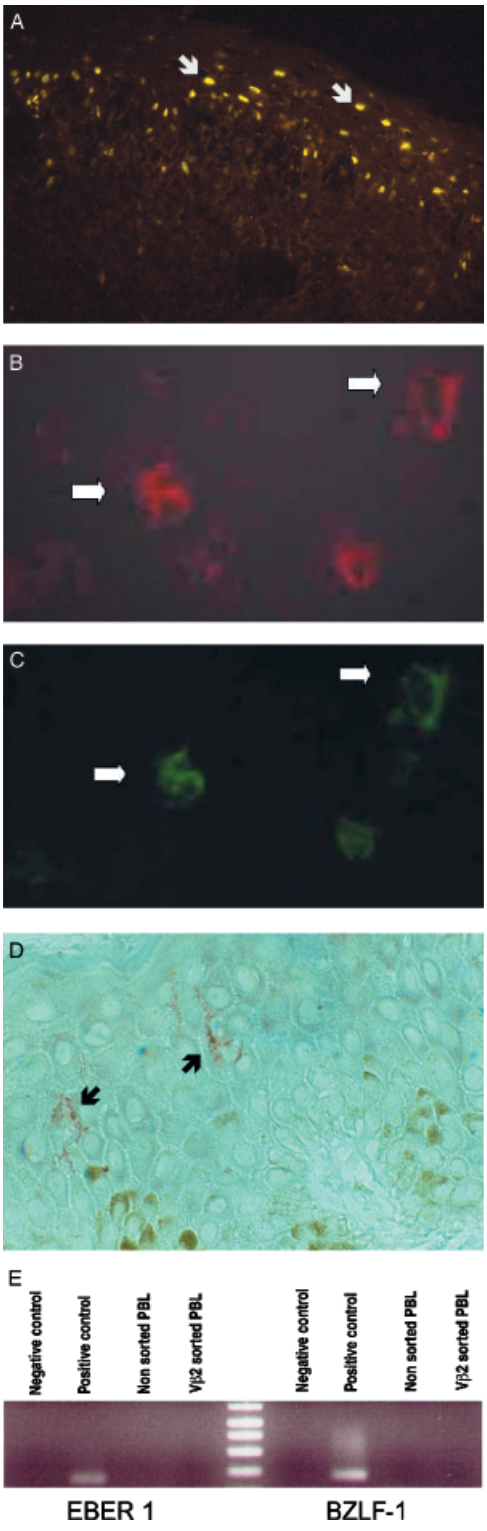

Figure 1
Detection of Epstein–Barr virus (EBV) in the cutaneous lesions and in the blood of cutaneous T cell lymphoma (CTCL) patients. Single staining of immunofluorescence with monoclonal EBNA2 antibody on cutaneous sections of lesional skin in CTCL (*A*). Double staining of immunofluorescence with CD1a monoclonal antibody (red, *B*) and monoclonal anti-EBNA2 on cutaneous sections of lesional skin in CTCL (green, *C*). The combined immunochemistry (CD1a monoclonal antibody) hybridization technique (EBER1 PNA probe) revealed CD1a-positive cells (red–brown color) with EBER RNA (nuclear dark-blue color) in epidermis (magnification $\times$ 40; 7 mm on scale bar represents 175 μm) (*D*). RT-PCR analysis of blood Vβ T cell clone RNA, note the absence of the virus (*E*).

whatever the stage may be. These EBNA2+ cells appear specific to CTCL skin lesions because of the lack of EBV expression in normal skin and in atopic dermatitis. Double labelling demonstrates that these EBNA2+ cells are CD1a+ cells, in other words Langerhans cells (LC). Moreover, we show that CD1a+ cells express EBER1 RNA, by a method combining immunochemistry and ISH, so this detection of EBV RNA within LC implicates a successful infection of LC by EBV.

This EBV infection raises the question of the potentially modified status of the LC: thereby such modified cells could attract T lymphocytes in the skin and induce their activation and chronic proliferation in the skin.

It is well known that the number of LC is elevated in the epidermis of CTCL (Meissner *et al*, 1993), decreased when remission occurs and increased as lesions recur (Braverman *et al*, 1987). The presence of the Pautrier microabscess, composed of large cluster of epidermotropic T cells surrounding LC in the epidermis and in dermal infiltrates shows that there are tight interactions between these cells (Chu *et al*, 1984). Our hypothesis is that due to this close apposition, the development of CTCL may be driven by LC triggering off CTCL cells growth through engagement of the T cell TCR. According to Berger *et al*, the growth of T lymphocytes of CTCL would be stimulated by CD1a+ autologous DC (Berger *et al*, 2002). Indeed, they report that T lymphocytes of CTCL could be grown in culture for 3 months when co-cultured with immature autologous DC. The T cell would retain the phenotype of the initial malignant clone, whereas the APC would be a mixture of immature and mature DC, the survival of both cell types being dependant on direct membrane contact.

In conclusion, our results show that EBV is absent in T lymphocytes from the blood of CTCL. But for the first time, we demonstrate the presence of this virus in the LC of the epidermis, with an expression of EBER1 RNA and EBNA2 protein. The virus could also play a role either in the modification of the functionality of these LC and in the activation and proliferation of T lymphocytes.

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Association of *TNF −238* and *−308* Promoter Polymorphisms with Psoriasis Vulgaris and Psoriatic Arthritis but not with Pustulosis Palmoplantaris

To the Editor:

Overexpression of tumor necrosis factor (TNF)-α is a central element in the pathogenesis of psoriasis vulgaris (PV) and psoriatic arthritis (PsA); however, the underlying mechanisms are poorly understood. Several recent studies with German patients found an association between the rare *A allele of the G → A single nucleotide polymorphism at position −238 of the *TNFA* promoter and psoriasis, particularly in patients with early disease onset (Arias *et al*, 1997; Hohler *et al*, 1997; Reich *et al*, 1999; Hohler *et al*, 2002; Reich *et al*, 2002). There is also evidence that carriage of the rare *A allele of another G → A single nucleotide polymorphism at position −308 of the promoter is decreased in patients with PsA (Hohler *et al*, 2002), although within this disease subgroup it may be increased in patients with a more progressive course of joint involvement (Balding *et al*, 2003). In light of the possible influence of *TNFA* promoter polymorphisms on cytokine production (Hajeer and Hutchinson, 2001), the increased formation of TNF-α in psoriasis could at least partially be genetically determined.

In this study, *TNFA −238* and *−308* genotypes were analyzed in 239 unrelated patients with PV, 43 patients with pustulosis palmoplantaris (PPP) without concomitant PV, and 135 control subjects according to published methods (Reich *et al*, 2002). All participants were Caucasians and were enrolled at the Department of Dermatology, University of Tartu, Estonia. Patients with PV were considered to have early-onset disease if skin symptoms occurred before 40 y of age, and late-onset disease if age at onset was $\geqslant$40 y (Henseler and Christophers, 1985). Disease severity was assessed at study entry by determination of the psoriasis area and severity index (PASI) (Fredriksson and Pettersson, 1978). PV patients were classified to have concomitant PsA ($n = 59$) if this diagnosis had been established by an experienced rheumatologist. Clinical deformities of the hands and/or feet consistent with PsA were seen in 23 of these patients, and nine patients had erosions of the hands and/or feet by radiographic assessment. Control subjects were recruited from among medical students, health care personnel, and patients presenting at the dermatological outpatient clinic with mild expression of either facial teleangiectasis or skin tags. The study was approved by the Ethics Review Committee on Human Research of the University of Tartu, and was conducted according to the Declaration of Helsinki protocols. Informed consent was obtained from all participants.

To evaluate deviation from the Hardy–Weinberg equilibrium, observed and expected genotype frequencies were compared by a Monte-Carlo goodness-of-fit test in PV and PPP patients and in controls. Odds ratios (OR) and exact 95% confidence intervals (CI) were calculated to compare genotype frequencies. Carriage rates of variant allele were investigated using the exact test by Fisher. To correct for multiple testing, a hierarchical test strategy was adhered to as described (Reich *et al,* 2002), and the respective nominal p values (p_{nom}) were adjusted according to Bonferroni–Holm (p_{adj}).

Absolute and relative *TNFA* genotype frequencies are shown in Table I. The genotypes were in Hardy–Weinberg equilibrium with the exception of the *TNFA −238* locus in the PPP subgroup ($p = 0.0118$). *TNFA* genotype frequencies in the control group were similar to those observed in other large European studies (Hohler *et al*, 1997; Reich *et al*, 2002).

Carriage of *TNFA −238*A* was significantly more common among patients with PV than among control subjects (23.8% *vs* 8.9%; OR 3.21 [1.60–6.84], $p_{adj} = 0.0012$), whereas carriage of *TNFA −308*A* was decreased (17.6% *vs* 29.6% in controls, OR 0.51 [0.30–0.86], $p_{adj} = 0.0360$). The latter finding was independent of *TNFA −238* as the difference between patients and controls remained similar after exclusion of *−238*A* positive individuals from the analysis (20.3% *vs* 30.9% in controls).

Abbreviations: CI, confidence interval; OR, odds ratio; p_{adj}, adjusted p value; p_{nom}, nominal p value; PASI, psoriasis area and severity index; PPP, pustulosis palmoplantaris; PsA, psoriatic arthritis; PV, psoriasis vulgaris; TNF, tumor necrosis factor